Workshop on Fluorescence Standards for Microarray Assays
December 10, 2002, 8:30-3:00 PM

Dr. Paul Smith (NIH/OD/ORS) "Operational principles of microarray readers"

- Chemically modified, glass, plastic, etc. with free amine groups to bind DNA or proteins.
- cDNA you are looking for differences between normal and abnormal DNA. Glass slide is prepared with library modules.
- Printer could generate 48 slides at a time → 15,000 spots per slide; 1-10 nl per spot.
- Detecting a few hundered molecules per spot.

What are the incorporation differences between different fluors?

- Do raster scanning of the spots. Have to account for an acceleration and deceleration phase of the stage, so that the slide is moving at the same velocity for each slide. Slew rate is 100 mm/s.
- All incoming and outgoing light is passed through a dichroic filter.
- Each pen has a slightly different profile on the array. A mask is generated based on the profile. **Distinguishing between background and actual signal then becomes critical. A sample with varying spot loading can help determine this.**
- Problems with photobleaching of fluorescent dyes.

Discussion:

- A stable fluorescence standard that is applicable to the visible region of the spectrum and that can be quantitated.
- Need a standard to measure performance between technologies/platforms.
- Linearity measure and ability to distinguish between noise and signal.
- Spatially uniform intensity reference.
- Ground truths on the ratio of expression between wavelength regions.
- The filters are important, because one needs to avoid "bleed-over" for quantitation. The Dyes typically used are FITC, Cy3 and Cy5.
- Flow cytometry has already tackled many of these questions and problems, and should be looked at to address these problems.
- Laser power is another concern for photobleaching, so that has to be a consideration for any standards in this area. Agilent has studied this, and has seen 0.25-0.5% photobleaching per scan on Cy3 and Cy5 and with 20 mW laser power and a 5 pixel scan area.

John Corson (Agilent Technologies) "Use, Design and Performance of Microarray Readers"

- The purpose of the reader is to measure the concentration of nucleic acid sequences.
- They enable highly parallel screening.
- True purpose is to generate an accurate, reproducible spatial map of the data.
- The image file generated is usually a TIFF file, and the data is extracted from the TIFF image.
- The fluorescent markers emit distinct spectra when illuminated by specific wavelengths of light.
- The excitation spectra tend to be very close, even overlapping the emission spectra.

- Dichroic filters separate the excitation and emission photons
- Scan lens should have a large numerical aperture with respect to the focal length to collect the most light.
- Detectors should have high quantum efficiency.
- It is critical to define the limits of the scanner accuracy, since this is necessary to distinguish signal from noise.
- Performance parameters: scale factor (how does intensity scale to concentration of fluor?); detection limit; signal to noise ratio; field uniformity; spectral crosstalk
- Known dye concentration standards permit you to determine the scale factors (counts per pixel per chromophore per square micron). Data can then be put into terms of chromophore density
- Repeated scans and rotated arrays permit you to separate variations due to the scanner and the array.
- What is the thickness of the material spotted on the glass? → Smaller than the wavelength of the light.
- Detection limit is a direct measurement of the sensitivity. High levels of intensity have noise that is proportional to the square of the number of photons. As signal levels decrease you have a noise floor that is dependent on the shot noise from the background light. Glass background photons are dependent on the platform of the scanner. Chemical background photons are dependent upon the array. Upper limit of example had a S/N of ~100 and the noise floor was ~3.
- Non-repeatability noise can be converted to a S/N and it is roughly equal to 1/ (S/N).
- Scan Field Non-Uniformity can dominate the error of single color S/N → Can be measured with a uniformly coated dye, but provides combined information about field non-uniformity and spatial noise.
- Crosstalk: With only green dye, one can plot the amount of red signal to green signal to get the information about the green to red crosstalk.
- Dynamic range between the scanners is another important parameter that one should examine.
- Feature extraction software should also be examined.

Dr. Zygmunt Gryczynski (Assistant Director, Center for Flourescent Spectroscopy, University of Maryland) "Fundamentals of fluorescence from surface confined materials"

- The center is funded by NIH, National Center for Research Resources (NCRR)
- Characteristics: spectrum, fluorescence lifetime, quantum yield, fluorescence polarization and quenching
- Steady-state versus time-resolved fluorescence
- How difficult are measurements on a surface? → Reflection and refraction become important; small volume, high intensity illumination and low photostability are characteristics of microarrays; for single-molecule-detection, photostability is necessary, because you only get ~10^8 photons per second
- What is the limiting factor for fluorescence detection? → depends on number of molecules, excitation light intensity and solvent conditions
- Standards: thin layer, good signal level, photostable, easy to use under various conditions

- Problems: Interaction with the surface, oxygen quenching, sample heating (affects quantum efficiency)
- Dye embedded polymers, dye deposited on solid surfaces, or dye on monolayers
- Self-absorption, self–quenching, and polarization of the sample are critical concerns of standards.
- Slides with silver particles can increase the fluorescence and compete with quenching. Metal-enhanced fluorescence. High quantum yields with shorter fluorescent lifetimes. This can also direct fluorescence towards the detector.
- Have used monolayers over the silver particles.
- Should and can quantum dots be used for standards?

Youxiang Wang (Full Moon Biosystems) "Scanner calibration and Validation Slides"

- For diagnostics, protocols must meet the certification standards of the ISO, GMP and FDA.
- Calibration slide requirements: High Sensitivity (low background); Cover a wide dynamic range; stable; accurate; great spot morphology, scanner stability
- Use a surface stabilized reagent.
- Important to balance the 2 fluors when doing differential expression experiments.
- Alignment can be checked using spots that have 2 fluors and whether the spots from both channels align.

John Corson (Agilent Technologies) "Performance Calibration Standards"

- It is important to be platform independent.
- The standards will not necessarily make the data better, but the scanner will be evaluated and what will be provided is a confidence in the data measured.

What about spot size and shape, and how much of that is software and hardware related?

- Performance standards: Detection Limit; Log(Ratio) Error; Single Color S/N; Spectral Crosstalk;
- Rotating the slide with a standard is critical for evaluating the scanner.
- Gradient slide with fiducials for alignment would be useful for 180^{o} rotations.

There seems to be a misconception between physical standards and standard protocols.

Greg Loney (Affymetrix)

- Interested in optical standards.
- 532 nm excitation/570 nm emission (phycoerythrin)
- 20 $\mu W/\mu m^2$ laser excitation power
- Scanner uses: field uniformity; absolute fluorescence measurement; detector sensitivity; detector linearity
- Not interested in crosstalk, since Affymetrix is a single color platform
- Zinc chloride and other inorganics may help as a standard. There was discrepancy about this, because quantitation would not be as meaningful with these fluors.

Simon M. Lim (Duke University Medical Center) "Need for a Standard – Genomic Medicine View"

- Intra- versus inter-laboratory measurements
- QA/QC charts are used to determine which laboratories may have problems
- Standard reference materials for clinical laboratory proficiency testing
- Inter-laboratory agreement is poor for microarrays, Isaac Kohn *Bioinformatics*, 18
- Technical variability has to be much smaller than biological variability, which is of interest.
- CAMDA'02 Conference: Technical variability is much larger than biological variations; SOPs could help to reduce these technical variations; fluorescent calibration should help normalization strategies; http://camda.duke.edu
- Jack Pronk, *JBC* **227**:37001-37008 (2002)
- Spike-ins; Compare to a golden standard: RT-PCR; Mix Experiments (Serial Dilutions)

Siobhan Pickett (Axon Instruments) "Microarray Scanner Performance Validation Standards" (Conference Call)

- Detection Limits and Calibration: Interested in matching the dyes of interest
- Values should be in molecules per unit density (area squared) → Standards should cover the whole dynamic range of the instrument.
- Stability: Should allow for multiple scans
- Batch consistency
- Field uniformity: Use ultra-flat glass (±1 μm); well distributed spots with a strong signal; user scans forward and reverse to show scan-to-scan reproducibility
- Imaging resolution: precision line-pair patterns would allow for resolution measurements; varying lines size and spacing; photostable; potentially a mimic of the actual dye to be used, but not necessarily that dye
- Long-term signal output benchmark: Access and calibrate signal output over life; insensitive to surface variation; photostable; mimic spectral properties of the actual dye

Dile Holton (Perkin Elmer Life Sciences) "Proposed Specifications for Microarray Scanners"

- PE provides a confocal laser scanner
- Assumptions: standards will be made around standard fluorescent dyes; 16 bit TIFF images will be used; Image analysis will be performed with array informatics tools
- Key Considerations: S/N (5-10); signal or brightest spots (maximum dynamic range; set usable/observable dynamic range); Noise adjustments (machine noise; lab process noise; zonal processing)
- Detector/Excitation Source Balance; pixel size; speed; optical focus; automatic adjustments
- What is a good microarray image? → Performance validated with standard materials and methods; standardized outputs for easy downstream analysis.

Edward Pope (Matech)

- There will probably not be one standard that will be able to address the needs of everyone.

- There is no such thing as a stable organic dye → The organic dyes are often sensitive to temperature, pH, solvent (reversible) and photobleaching and chemical modification (irreversible).
- The standards have to be a GMP manufactured product and the devices should be registered with the FDA.
- The standards have to have less noise than the variation that you are trying to measure
- Geometric uniformity is necessary.
- Lanthanide-doped glasses are potential fluorophore standards.
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- Photolithography can be used to etch 125 μm holes in a thin metal mask that has been evaporated onto a 100 nm thick fluor-doped layer to make a standard.

Charles Epstein (Aventis Genomics, Inc.) Not Present

Gary Kramer (NIST, ACD) & Adolfas Gaigalas (NIST, Biotechnology Division) Summary and Discussion

- Proposed an evaporated metal gradient in a sandwiched slide similar to the Matech proposal, but this will allow an intensity gradient
- There are about 2 sizes of slides with different thickness of glass. (ISO microscope slide specs: ISO 8037-1:1986 slide dimensions; ISO 8037-2:1997 slide properties; ISO 80255:1997 cover slips)
- Spot sizes vary between 20 μm and 100 μm
- The intensity and stability standards are important
- Resolution targets should be different
- Users are used to spots and they should be given spots
- Need to get the specifications of each of the microarray scanners and then NIST will come back with a proposal.
- Set up a timeframe to get information to NIST (Before the holidays are over). mailto:gary.kramer@nist.gov
- Regular interaction was proposed to develop this group into a working group
- A preliminary proposal will be presented before PittCon